
CONGRUENCE AND CONFLICT BETWEEN MOLECULAR AND REPRODUCTIVE CHARACTERS WHEN ASSESSING BIOLOGICAL DIVERSITY IN THE WESTERN FANSHELL *CYPROGENIA ABERTI* (BIVALVIA, UNIONIDAE)¹

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ABSTRACT

Organisms with complex life histories and unusual modes of genome inheritance can present challenges for phylogenetic reconstruction and accurate assessment of biological diversity. This is particularly true for freshwater bivalves in the family Unionidae because: (1) they have complex life cycles that include a parasitic larva and obligate fish host; (2) they possess both a male and female mitochondrial genome that is transmitted through doubly uniparental inheritance (DUI); and (3) they are found in riverine habitats with complex hydrogeological histories. Examination of mitochondrial DNA (mtDNA) sequences, conglutinate morphology, and host fish compatibility of the western fanshell *Cyprogenia aberti* (Conrad, 1850) revealed significant character variation across its range. Although variation was correlated among the different data sets and supports discrete groups, these groups did not always correspond to geographically isolated populations. Two discrete mtDNA clades exist sympatrically within most *C. aberti* populations, and these same clades are also diagnosed by at least one morphological character, egg color. The surprisingly high genetic distance (14.61%–20.19%) between the members of these sympatric clades suggests heritance infidelity of the two different mitochondrial genomes. This hypothesis was tested and falsified. More general patterns in geography were observed in host fish compatibility. Populations of *C. aberti* from the major river systems differed in their ability to utilize fish species as hosts. These differences in reproductive traits, which are presumably genetically based, suggest that these populations are not ecologically exchangeable with one another and represent biological diversity not previously recognized within *Cyprogenia* Agassiz, 1852.

Key words: Central Highlands, coevolution, *Cyprogenia aberti*, double uniparental inheritance, mitotype, Unionidae.

Comparison among associated historical patterns is a powerful tool in evolutionary biology. Such patterns include organisms and geographic areas in vicariance biogeography (Rosen, 1978), the co-speciation of hosts and parasites (Hafner & Nadler, 1988), and congruence between gene trees and species trees (Goodman et al., 1979). Areas, organisms, and genes are analogous because each has a history that can be reconstructed via phylogenetic methods. Likewise, the affiliations between these patterns are analogous, for each pair has one entity that is connected to, and may track, the other because of causal interaction through evolutionary history (Page, 1993; Page & Charleston, 1998). Comparing lineage patterns in an evolutionary

association can provide insight into the biology of understudied organisms, direct research by focusing attention on meaningful patterns, reveal hidden biological diversity, and help identify the processes and mechanisms involved in speciation.

Although evolutionary associations may provide mechanisms for speciation, lineages (organism vs. gene vs. area vs. host) may or may not show congruent patterns. Pattern differences may reflect a unique history at one level that did not influence lineage diversification at another. An example of such incongruence would be the failure of a parasite to track its host after a host-switching event (Page, 1994). However, when data from different evolution-

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ary associations converge on a single pattern, it is a strong indication that the pattern represents the true species tree (Page & Charleston, 1998).

North American freshwater mussels in the family Unionidae offer an unusual opportunity to examine multiple associations simultaneously, because the greatest diversity occurs in areas with complex hydrogeological histories, their life cycle includes an obligate parasitic stage, and they possess an unusual mode of mitochondrial inheritance. The first two factors appear to play a major role in unionid speciation (Kat, 1984) and may be useful when assessing biological diversity (Roe & Lydeard, 1998; King et al., 1999; Roe et al., 2001). In contrast, mitochondrial genes are often used in phylogenetic reconstruction because most models of mitochondrial DNA (mtDNA) evolution consider neutral evolution and purifying selection as the major processes that shape sequence divergence. Thus, mtDNA variation should track species history. However, alterations in mitochondrial inheritance could affect phylogenetic inferences from these genes, making them incongruent with other lines of evidence (Hoeh et al., 1996, 1997).

The life cycle of North American unionids includes a larval stage that is obligately parasitic on a vertebrate host (for a general description of unionid life history, see Coker et al., 1921; McMahon, 1991). The parasitic larvae, called glochidia, are brooded inside the female mussel in a modified portion of the gill. Once mature, the glochidia must attach to a host to complete development. Generally, the host is a fish, and the glochidia encyst in the gill or fin epithelium of the appropriate host species. Unionids are typically able to utilize only a limited number of species as hosts. Glochidia that attach to a compatible host are encysted by migrating epithelial cells and subsequently transform into the juvenile stage (Arey, 1932; Rogers-Lowery & Dimock, 2006). Glochidia that attach to incompatible (non-host) species either fail to be encysted or are subsequently sloughed before transformation is complete (Arey, 1932; Meyers et al., 1980; O'Connell & Neves, 1999; Rogers & Dimock, 2003).

Relationships between mussels and host species may influence speciation rates and/or provide information for phylogenetic reconstruction. The degree of host specificity varies among unionid taxa (Hoggarth, 1992). Some host-parasite relationships are highly restrictive, so that a mussel species will only transform on a specific (sympatric) population within a host species range (Rogers et al., 2001; Eckert, 2003). In these instances, lineage associations between mussel parasite and fish host should be highly correlated.

Females of some host-specific mussel species (e.g., *Lampsilis cardium* (Rafinesque, 1820), *Villosa iris*

(Lea, 1829)) attract particular hosts by producing lures that mimic prey items of intended host (see Kat, 1984; Barnhart et al., 2008 and references within). Lures can be part of the female's mantle tissue or may take the form of conglomerates, aggregations of larvae (glochidia) and unfertilized eggs that are released from the female. When a fish attacks the conglomerate, it comes in direct contact with the glochidia, which increases the probability of infection. At the same time, the lure limits the diversity of fish that are likely to come into contact with the glochidia of the species and, therefore, affects the range of potential hosts. The evolution of lures may thus affect the evolution of host specificity. For example, change in display timing may alter the level of attractiveness to a host. Kat (1984) and Graf (1997) suggest that this change in fish hosts might result in the formation of new unionid species via sympatric means.

Another evolutionary association in unionids occurs between the organism and its geographic range (area). Similar to freshwater fishes and crayfishes, unionids exhibit distinct faunal assemblages and high endemism among river basins (Neves & Widlak, 1987; Vaughn, 1997). The greatest unionid diversity is found in ancient rivers with dynamic hydrogeological histories (Lydeard & Mayden, 1995; Neves et al., 1997). Many of these rivers have undergone reorganization during the cyclic glacial advances and retreats of the Pliocene and Pleistocene (e.g., Donn et al., 1962; Melhorn & Kempton, 1991) or experienced episodic tilt-block tectonics (e.g., Cox, 1994) during the Pleistocene and Early Holocene. These geologic events have profound evolutionary effects by restricting gene flow, causing allopatric speciation through vicariance, or creating opportunities for rapid radiations and secondary contact zones between once-isolated populations through stream-capture events (Roe et al., 2001; Kozak et al., 2006). Thus, understanding the historical geology of the drainages in which the study organisms occur is important when interpreting phylogenetic reconstruction. Phylogenetic patterns should reflect the hydrogeological history of the drainage basins when the organism is restricted to aquatic environments and has limited dispersal capabilities. There will be some exceptions to this prediction. In a dynamic environment with multiple, rapid cladogenetic events, area relationships and gene lineages may be in conflict with one another due to incomplete lineage sorting of ancestral polymorphisms. Incongruence between the unionid and area history also may occur when mussel species use hosts with high vagility.

Finally, unusual modes of genomic inheritance can complicate phylogenetic analysis and interpretation where the gene tree may not reflect the species tree.

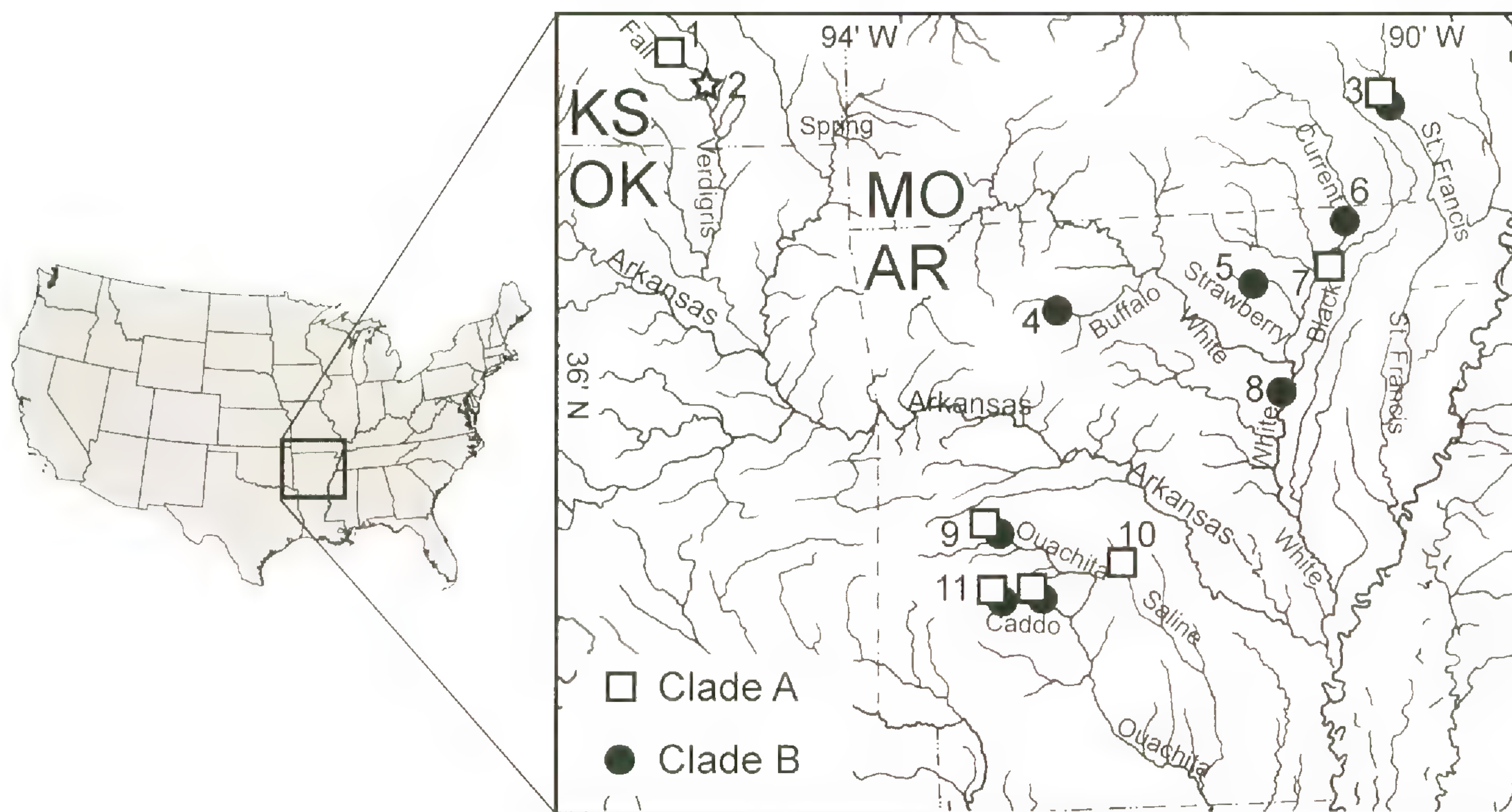


Figure 1. Distribution map of sampled *Cyprogenia aberti* localities. Squares and circles represent the two major genetic clades recovered in the phylogenetic analyses, clade A and clade B, respectively (see Fig. 3). A star represents the collection site on the Verdigris River that was used in a host identification study by Eckert (2003), but was not included in this phylogenetic analysis (but see phylogenetic analysis in Serb, 2006). Specific locality information is provided in Appendix 1 and Serb (2006). Collection sites are labeled by river name and number: Fall (1), Verdigris (2), St. Francis (3), Buffalo (4), Strawberry (5), Current (6), Black (7), White (8), Ouachita (9), Saline (10), and Caddo (11). States are labeled with abbreviations: Arkansas (AR), Kansas (KS), Missouri (MO), and Oklahoma (OK).

The Unionidae and two other bivalve families (Mytilidae and Veneridae) possess two different mitochondrial genomes (mitotypes) that are associated with gender (F for female and M for male) (Zouros et al., 1992, 1994; Hoeh et al., 1996, 1997; Liu et al., 1996; Passamonti & Scali, 2001). These sexual mitotype lineages remain separate via doubly uniparental inheritance (DUI) (Skibinski et al., 1994; Zouros et al., 1994), where the maternal mitochondrial genome (F-mitotype) is inherited by both male and female progeny from the mother, while the paternal mitochondrial genome (M-mitotype) is only transmitted from father to male offspring and housed in the male gonads. The resulting heteroplasmy is not necessarily limited to males. In both laboratory crosses and natural populations, low levels of DUI infidelity have been documented in *Mytilus* L. species (family Mytilidae) (Zouros et al., 1994; Stewart et al., 1995; Garrido-Ramos et al., 1998). This event is a role reversal of sexual mitotypes by either masculinization of the F-mitotype (M^f) or feminization of the M-mitotype (F^m) (as described by Hoeh et al., 1997). The result is that two lineages of the functional mitotype (e.g., F and F^m) co-exist in a population. These events can confound the interpretation of phylogenetic analyses when non-orthologous genes (F vs. F^m) are inadvertently compared (see Hoeh et al., 1996, 1997), so that the resulting gene tree would not represent the species tree.

CYPROGENIA ABERTI. A CASE STUDY

The western fanshell *Cyprogenia aberti* (Conrad, 1850) is the only congener of the federally endangered *C. stegaria* (Rafinesque, 1820) (U.S. Fish & Wildlife Service, 1990). These two species occur in the Central Highlands of North America, with *C. aberti* found in the Interior Highlands west of the Mississippi River and *C. stegaria* in the Eastern Highlands east of the Mississippi River. The Central Highlands are drained by high-gradient, clear streams and are isolated by low-gradient habitats that are typically silt loaded. The two Highland provinces contain a diverse, well-studied aquatic fauna and many endemic taxa, including fishes (Wiley & Mayden, 1985; Mayden, 1987, 1988), amphibians (Routman et al., 1994), and crayfishes (Crandall & Templeton, 1999).

Cyprogenia aberti is endemic to rivers draining the Ozark Plateau and the Ouachita Highlands of the Interior Highlands. These include upland portions of the Arkansas, Ouachita, White, and St. Francis river systems in Arkansas, Kansas, Oklahoma, and Missouri (Fig. 1). Many *C. aberti* populations are isolated by geography between rivers and within river systems. For example, populations in streams of the upper Arkansas, Black, and White rivers are all separated from one another by the lower-elevation, low-gradient portions of these systems.

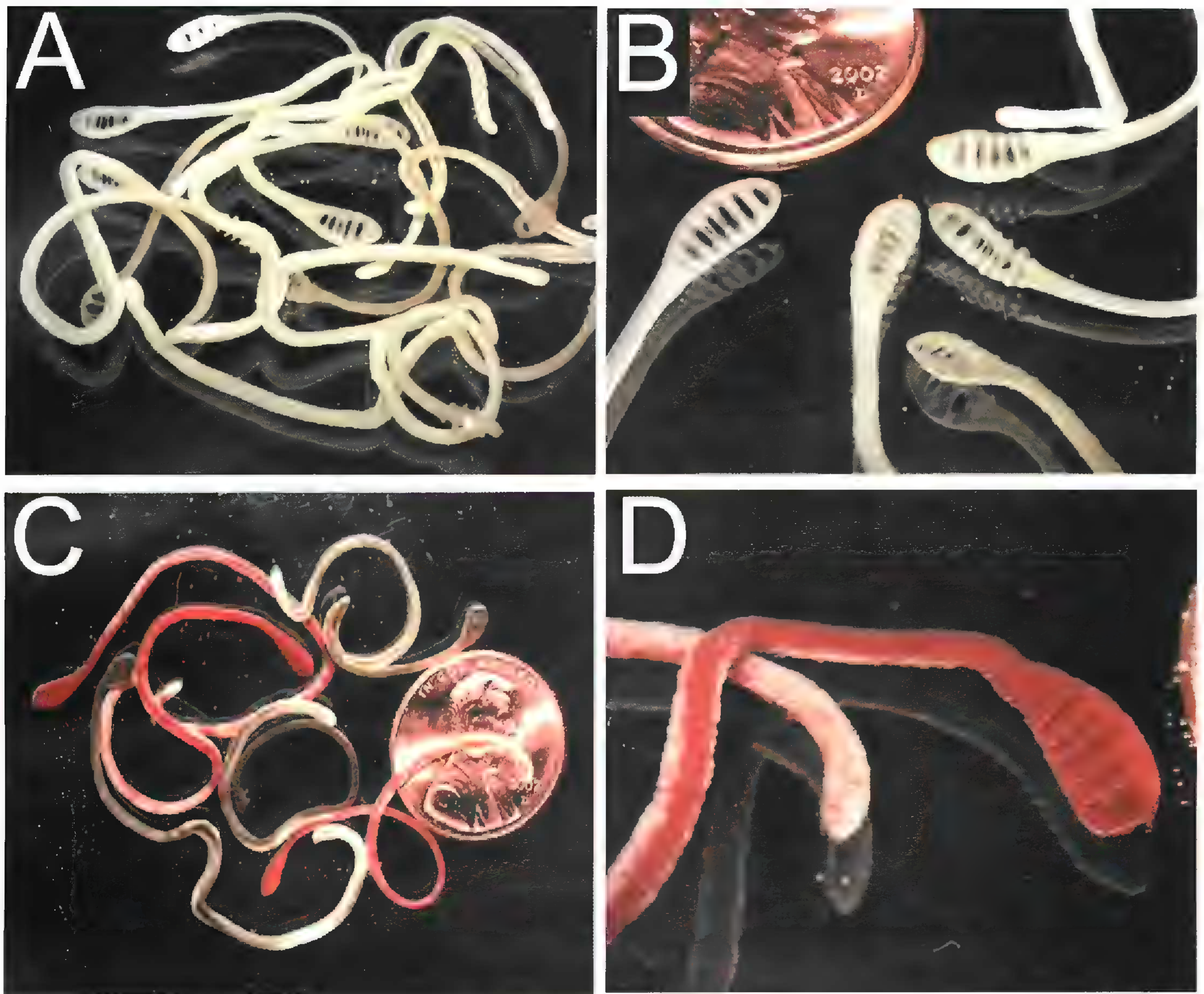


Figure 2. Variation in cyprid morphology. Color of the unfertilized eggs and the structure of cyprid lure differs among *Cyprogenia aberti* females. —A–B. White cyprids were produced by a female collected in the Verdigris River, Kansas. Similar cyprid morphology is produced by Fall River females. —C–D. Red and brown cyprids were produced by two sympatric females collected in the St. Francis River, Missouri.

Geography would appear to be the best predictor of how populations of *Cyprogenia aberti* would be related to one another due to their disjunct distribution. However, recent genetic investigations of *C. aberti* using mtDNA sequence data indicate that geography only partly explains the phylogenetic pattern (Serb, 2006). Although the phylogenetic clades correspond to drainage, this pattern was replicated within *C. aberti*, where two genetically distinct sympatric lineages were identified within the Ouachita River system as non-sister clades (Serb, 2006). If the mitochondrial gene tree accurately describes the species tree, these data suggest that there are as many as five distinct evolutionary lineages within *C. aberti* (Serb, 2006). To test the accuracy of this biodiversity assessment, we compared molecular, geographic, and reproductive characters.

Reproductive traits employed in attracting a fish host, such as lure morphology, presumably have a genetic basis and may provide additional data for

determining the species tree. In *Cyprogenia*, cyprid coloration varies among species and populations. For example, *C. stegaria* typically produces red worm-like cyprids (Jones & Neves, 2002). Among populations of *C. aberti*, red cyprids have been documented from the St. Francis River (Chamberlain, 1934), while white cyprids were observed in the upper Arkansas River (Barnhart, 1997) (Fig. 2A, B). Most recently, Eckert (2003) identified populations in the Ouachita and St. Francis rivers that contain females that produce either red or brown cyprids (Fig. 2C, D). Cyprid color is the result of pigmentation in the sterile (structural) eggs (Barnhart, 1997; Eckert, 2003). The red and brown forms are pale when immature but darken during development (Barnhart, pers. obs.).

The goal of this study is to better understand the types of data needed to accurately assess biological diversity in organisms with complex evolutionary histories. We explore how three evolutionary associ-

ations of a North American unionid, *Cyprogenia aberti*, relate to one another: reproductive traits associated with host preference, geographic distribution, and mitochondrial gene variation. Specifically, we compare the geographic distribution and reproductive traits with a mitochondrial gene tree derived from previous work (Serb, 2006).

MATERIALS AND METHODS

Thirty-one individual specimens of *Cyprogenia aberti* representing 11 different localities were collected across its extant range in Arkansas, Kansas, and Missouri. Individuals are identified with a unique museum number and river location. The following river and geographic notations are used. "White" refers to the upper White River above the confluence of the Black River and its tributaries (Strawberry, Current, and Buffalo rivers). The upper St. Francis River is included in the White designation, because the two rivers have similar fish faunas attributed to interchanges between headwaters (Cross et al., 1986). "Arkansas" refers to the upper Arkansas River and its tributaries in Kansas (including the Fall and Verdigris rivers). "Ouachita" refers to the Ouachita, Caddo, and Saline rivers in the Ouachita Highlands. Rivers of the Interior Highlands include all drainages of the Ozark Plateau and Ouachita Highlands. Figure 1 is a map of rivers and localities in this study.

DNA SEQUENCING

Genomic DNA was extracted from the *Cyprogenia aberti* specimens and six outgroup taxa also within the Unionidae: *C. stegaria* (eastern fanshell), *Dromus dromas* L. Lea (dromedary pearly mussel), *Lampsilis ornata* Conrad (southern pocketbook), *L. siliquoidea* Barnes (fatmucket), *Obliquaria reflexa* Rafinesque (three-horn wartyback), *Ptychobranhus fasciolaris* Rafinesque (kidneyshell) (Appendix 1). Mantle tissue along the shell was used in standard phenol/chloroform extraction with ethanol precipitation (Roe & Lydeard, 1998) to reduce the probability of non-orthologous mitotype contamination from the male gonad.

DNA amplification and sequencing of two mitochondrial gene portions were performed as described in Serb (2006). A 600-bp region of the first subunit of cytochrome oxidase *c* gene (CO1) was amplified and sequenced using primers LCO1490 and HCO2198 (Folmer et al., 1994) or a modified HCO2198 primer (Graf & Ó Foighil, 2000). A 700-bp region of the 5' end of the first subunit of the NADH dehydrogenase gene (ND1) was amplified and sequenced using primers Leu-uurF and NIJ-12073 (Serb et al., 2003).

Purified polymerase chain reaction (PCR) products were used as a template for cycle sequencing reactions with the ABI Prism Big Dye Terminator kit (Applied Biosystems, Foster City, California). Sequencing reactions were visualized on an ABI 3100 automated sequencer.

A male mitotype from *Cyprogenia aberti* was isolated and sequenced for phylogenetic analysis. Testis tissue was dissected from an ethanol-preserved *C. aberti* male from the Black River, Missouri (see Appendix 1). Total RNA was isolated from gonad tissue using TRIZOL (Invitrogen, Carlsbad, California), followed by phenol/chloroform extraction and ethanol precipitation. RNA was used as a template for complementary DNA synthesis in reverse transcriptase-PCR with a poly-T primer. Complementary DNA was quantified and used in subsequent PCR to amplify the male mitotype copy of CO1. A 550-bp region of the male copy of CO1 was amplified and sequenced using primers UNIOCMCOIF (Curole, 2005) and HCO2198, by means of the following thermal cycling conditions for 34 cycles: denaturing at 94°C for 40 seconds, annealing at 45°C for 60 seconds, and extension at 72°C for 90 seconds.

CONGLUTINATE PHENOTYPES

Six to eight gravid females of *Cyprogenia aberti* were collected from the Fall (Arkansas system), St. Francis, and Ouachita rivers. These females were brought back to the laboratory, where conglutinate release was induced by raising water temperature from 5°C to 20°C over a period of seven hours. Maturity of glochidia and conglutinate color were determined, which allowed for individuals to be designated as known phenotypes. All animals were preserved in 95% ethanol. DNA sequencing was performed as described above for correlation with conglutinate phenotype.

PHYLOGENETIC ANALYSES

All double-strand DNA sequences were visually aligned unambiguously and were converted into amino acid sequence to check the accuracy of the nucleotide sequence in the data editor BioEdit (Hall, 1999). For each protein-coding gene, an evolutionary model was determined using the log likelihood ratio test (LRT) as implemented in Modeltest version 3.0 (Posada & Crandall, 1998). Pairwise sequence divergences were calculated between all taxa using the appropriate evolutionary model. Mean sequence divergence was calculated within and between phylogenetically defined clades.

Aligned sequences were analyzed phylogenetically under two optimality criteria: maximum parsimony (MP) using PAUP* version 4.0b10 (Swofford, 2002) and Bayesian using the program MrBayes version 3.0 (Huelsenbeck & Ronquist, 2001). All MP analyses employed a heuristic search of 100 random-order addition of taxa and tree bisection-reconnection (TBR) branch swapping. Only minimum-length trees were retained and zero-length branches were collapsed. Support for the individual nodes of the resulting phylogenetic hypotheses was assessed by bootstrap values using the FAST step-wise addition option (2000 replicates) in PAUP*, and decay index values (Bremer, 1994) were calculated with TreeRot version 2c (Sorenson, 1999). Outgroup taxa within the Unionidae were chosen based on a molecular phylogeny of the tribe Lampsilini using CO1 and 16S ribosomal RNA (rRNA) sequence data (Roe, 1999). Trees were rooted using the species *Lampsilis ornata*. MP analyses comparing M- and F-mitotypes were based on the CO1 gene alignment. Trees were rooted using the M-mitotype copy of CO1 from *L. teres* Rafinesque (GenBank accession number AF406794).

A Bayesian analysis of the concatenated mitochondrial genes was conducted using six data partitions: first, second, and third codon positions for two protein-coding genes. Four Markov chain Monte Carlo (MCMC) simulations were run simultaneously using a random starting tree for 3×10^6 generations to estimate the topology and posterior probability for node support. Trees were sampled every 100 generations, and the number of trees discarded (burn-in) was determined by plotting the log likelihood scores of all saved trees versus generation time. Only trees with likelihood scores after stationarity was achieved were retained for inclusion in the consensus tree. Node support was determined by the frequency at which a particular clade occurred within all trees retained after the burn-in trees were discarded.

RESULTS

Conglutinates from Fall River (1, cf. Fig. 1) *Cyprogenia aberti* females were all white in color, similar to previous observations of specimens from the upper Arkansas system (Fig. 2A, B; Barnhart, 1997; Eckert, 2003). Females from the St. Francis (3, Fig. 1) and Ouachita (9, Fig. 1) rivers produced either red or brown congrutinates (Fig. 2C, D).

Sequence alignment of the CO1 and ND1 gene portions yielded 1126 bp, containing 402 polymorphic sites, 261 of which were phylogenetically informative under MP. The MP analyses conducted on these concatenated sequences resulted in 7476 equally most parsimonious trees (MPT) of 734 steps

(Fig. 3). None of these trees supported a monophyletic *Cyprogenia aberti* due to the placement of two American Eastern Highland species, *C. stegaria* and *Dromus dromas*, within the *C. aberti* clades. In the Bayesian analysis, stationarity was achieved after 650,000 generations, and 8500 trees were discarded as burn-in. The 50% majority-rule consensus tree was based on the remaining 21,437 trees ($-\ln L = 4895.566$) and was similar to the MP topology (Fig. 4).

In both phylogenetic analyses, *Cyprogenia* form two major clades (A and B). Clades within the large A and B clades can be defined generally by river system membership of the *C. aberti* individuals (designated as Ouachita, White, or Arkansas) creating parallel geographic structure. Both clade A and clade B contain Ouachita (Ouachita [9], Saline [10], Caddo [11], sites, cf. Fig. 1) and White (St. Francis [3], Buffalo [4], Strawberry [5], Current [6], Black [7], White [8], sites, cf. Fig. 1) clades. Sympatric individuals from these two drainages segregate by congrutinate color: red congrutinate producers have clade A membership, while brown congrutinate producers belong to clade B (Fig. 2). Clade A also includes white congrutinate producers from the Fall River site (1, cf. Fig. 1) and the Verdigris River (see Serb, 2006) in the Arkansas Basin.

Only one node differs between the MP and Bayesian topologies. In the MP topology, the red congrutinate-producing Ouachita clade (Ouachita, Caddo, Saline exemplars) is the sister group to the White/*Cyprogenia stegaria* + Arkansas clade (2 decay index; less than 50% bootstrap support), while the Bayesian topology places the red congrutinate-producing Ouachita clade as the sister group to the White/*C. stegaria* clade (83 posterior probability). This topology places all red congrutinate producers together (Ouachita, White, and *C. stegaria* sites) and the white congrutinate producers in the Arkansas River as the basal group (Fall River site, Fig. 4).

Sequence divergence was calculated for each river drainage and phylogenetically defined groups within a single river (Table 1). Because CO1 and ND1 gene portions were determined by Modeltest to have two different sequence evolution models, we calculated sequence divergence for each gene separately. Evolutionary models for CO1 and ND1 were variations of the general time reversible (GTR) model: the Tamura-Nei model (TrN+ Γ) with an adjustment for among-site rate heterogeneity ($\alpha = 0.2428$) for CO1, and the transversion model (TVM+I) with a parameter for the proportion of invariant sites ($I = 0.5211$) for ND1. Generally, sequence divergence within a phylogenetically defined clade was low, less than 1%. These groups corresponded to congrutinate color (red, brown, or white) within *Cyprogenia*. In contrast,

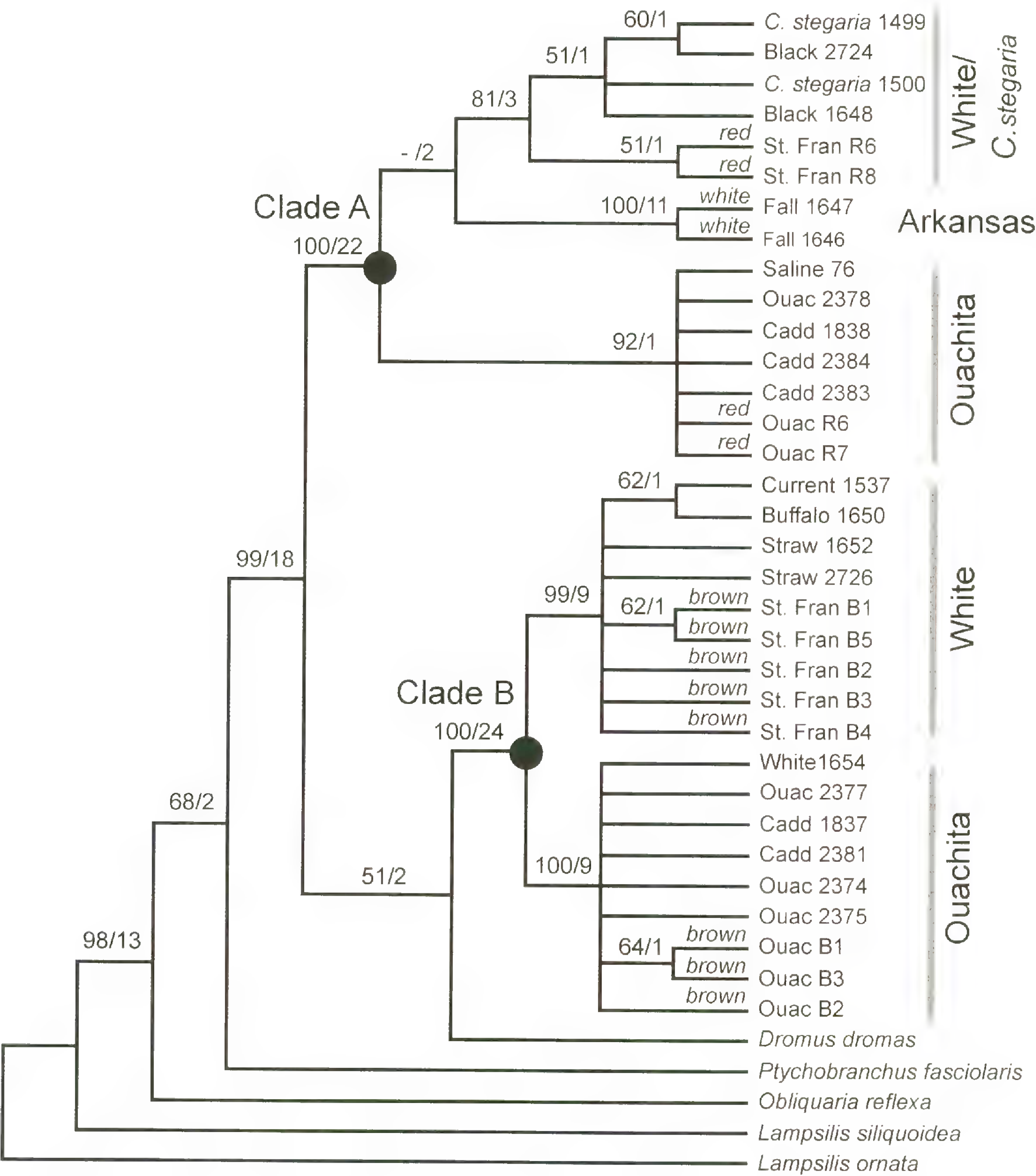


Figure 3. Strict consensus of 7476 trees recovered in maximum-parsimony analysis of the mtDNA sequence data set (tree length [TL] = 734; consistency index [CI] = 0.683; retention index [RI] = 0.895). Numbers above branches represent bootstrap support greater than 50% (2000 replicates) followed by decay index values. The two major *Cyprogenia* clades are labeled as A and B. Smaller clades are labeled by river drainage along terminal branches. Individuals with known conglutinates color are labeled (red, white, or brown) on terminal branches. Species currently recognized as *C. aberti* are listed by University of Alabama Unionid Collection museum number and river locality. River abbreviations are: Cadd = Caddo; St. Fran = St. Francis; Straw = Strawberry; Ouac = Ouachita. Other species are listed by species name in italics; *C. stegaria* specimens include museum accession numbers (see Appendix I).

when means were calculated for drainages that included mitochondrially distinct, red and brown conglutinate producers, these values increased dramatically. Within a single location in the St. Francis River that contained red/brown conglutinate variation, individuals were more than 14% divergent (averages,

COI = 14.6%; ND1 = 17.8%). These values were slightly higher in the Ouachita riverine system (averages, COI = 15.3%; ND1 = 20.2%). Again, these values are estimated from phylogenetically distinct genotypes (in A and B clades) found among sympatric individuals in two different river drainages

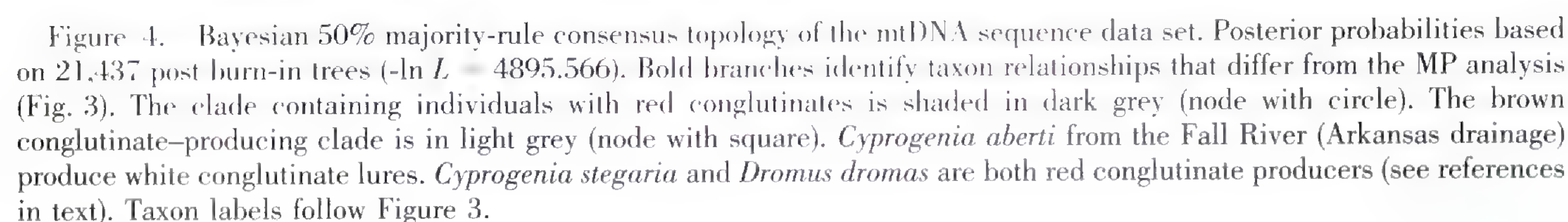


Table 1. Mean sequence divergence within drainage lineages (e.g., Arkansas), within conglutinate color lineages (e.g., red or brown), or between conglutinate color lineages (e.g., red vs. brown) as identified through phylogenetic analysis (see Fig. 3). Specific models of sequence evolution were determined for each mitochondrial gene portion: Tamura-Nei (TrN+ Γ) corrected distances for the first subunit of cytochrome oxidase *c* gene (COI); transversion (TVM+I) corrected distances for the first subunit of the NADH dehydrogenase gene (ND1).

	COI	ND1
	TrN+ Γ	TVM+I
White/ <i>C. stegaria</i>	0.47%	0.54%
Arkansas	0.08%	0.82%
St. Francis		
Red	0.19%	0.35%
Brown	0.66%	0.21%
Red vs. brown	14.61%	17.76%
Ouachita		
Red	0.61%	0.47%
Brown	0.15%	0.09%
Red vs. brown	15.33%	20.19%

(Ouachita and St. Francis, within the White system) and not monophyletic entities.

Sequence alignment of the M- and F-mitotype copies of the COI gene yielded 529 bp, containing 253 polymorphic sites, 167 of which were phylogenetically informative under MP. The MP analyses resulted in four equally MPTs of 456 steps. None of the MP topologies placed the *Cyprogenia aberti* M-mitotype within the F-mitotype *Cyprogenia* clade (100% bootstrap support; Fig. 5). In addition, the dissimilarity between the amino acid sequence of the M- and F-mitotypes suggests that they are non-orthologous gene copies (data not shown; available from first author).

DISCUSSION

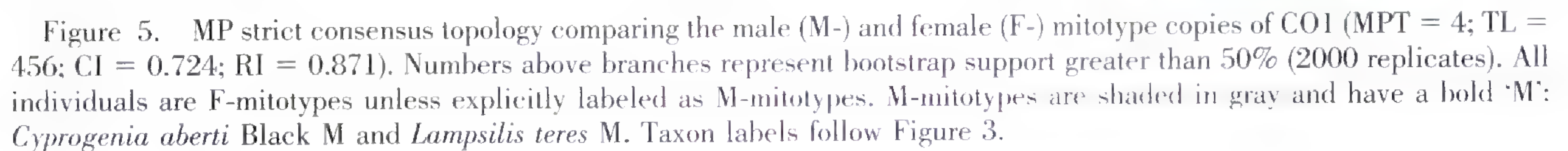
CONGRUENCE BETWEEN MITOCHONDRIAL PHYLOGENY AND REPRODUCTIVE TRAITS

This study attempted to test whether a mitochondrial gene tree with a replicated geographic pattern is an accurate assessment of the species tree in an organism with a complex life history. We compared the mitochondrial gene topology to life history trait patterns in *Cyprogenia aberti*. First, we examined a reproductive trait potentially key to the host-parasite association, conglutinate color. Variation in conglutinate lure color corresponded to the different mitochondrial lineages of *C. aberti*. All *Cyprogenia*

produce worm-like conglutinates that are either white, red, or brown (Fig. 2). Each color morph appears to match clades defined by the mitochondrial analyses, thus supporting the mitochondrial gene phylogeny with an independent data set. Clade A has mostly red conglutinate producers, with the exception of white conglutinate producers of the upper Arkansas system (Fall River site). In contrast to clade A, clade B consists of brown-producing females, with the exception of *Dromus dromas*. Conglutinate lures produced by the closely allied *D. dromas* are morphologically distinct. On average, they are shorter, wider, and flatter than *Cyprogenia* conglutinates and, unlike *C. aberti* of clade B, are red in color when mature (Ortmann, 1912; Jones & Neves, 2004).

Other reproductive characters may be important in estimating biological diversity and understanding speciation mechanisms of *Cyprogenia aberti*. One trait is host fish preference. Although all *C. aberti* populations use small, benthic-dwelling species in the darter genera *Etheostoma* Rafinesque and *Percina* Halderman, host use varies among river basins (Barnhart, 1997; Eckert, 2003). Eckert (2003) found variation in host preference among four *C. aberti* populations: Spring (in Kansas) and Verdigris (2, Fig. 1) rivers of the Arkansas system, Ouachita River (9, Fig. 1), and St. Francis River (3, Fig. 1). All *C. aberti* populations examined had high transformation success (% of glochidia that complete development) on *P. caprodes* Rafinesque, a large darter species that is widely distributed, but not always locally abundant, across the range of *C. aberti* (Robison & Buchanan, 1992). In addition, each *C. aberti* population used at least one other host of more limited distribution. For example, *P. phoxocephala* Nelson, a host for Verdigris River *C. aberti*, co-occurs with *C. aberti* only in the upper Arkansas drainage (Page, 1983). Significantly, allopatric pairings of mussel populations and host species showed poor transformation success. However, differences in host compatibility between red and brown conglutinate-producing individuals were not evident (Barnhart, 1997; Eckert, 2003; Barnhart & Eckert, unpubl.). Difference in host compatibility among river drainages provides additional evidence for the distinction among genetically defined allopatric lineages of *C. aberti* and not the sympatric lineages in the Ouachita and White rivers. Change in host utilization may be an important factor in diversification of *C. aberti* lineages (see below).

Second, we looked for direct evidence of gene tree/species tree conflict by comparing two different mitochondrial lineages in *Cyprogenia aberti*. A copy of COI from the *C. aberti* M-mitotype was included in phylogenetic analyses to test the hypothesis that the



pattern found in *C. aberti* is not due to a role-reversal event. Instead, the observed genetic diversity found in *C. aberti* mitochondrial sequences arose within the *Cyprogenia* F-mitotype lineage. These data support recent evidence that DUI has been operating at a high level of fidelity in Unionoidea for over 100 million years (Curole & Kocher, 2002; Hoeh et al., 2002; Walker et al., 2006).

EXPLANATIONS FOR DIVERSITY PATTERNS

Examination of the mitochondrial sequence data in conjunction with observed phenotypic diversity within populations of *Cyprogenia aberti* suggests existence of multiple evolutionary entities (a species complex). In this section, we present several possible processes that may have produced this observed diversity. One mechanism is that the two lineages (A and B) formed in allopatry, and the river systems where they co-occur represent a secondary contact zone (clades A and B in Figs. 3, 4). This hypothesis depends on both vicariance events and subsequent range expansions that may have been driven by abiotic changes during the Pliocene or Pleistocene. As glacial advances during these epochs altered the ecology and geology of the Central Highlands region (see Mayden, 1987), these changes impacted distribution patterns of the biota. Fluctuations in climate, water levels, and reorganization of drainage patterns within the Highlands could isolate and displace populations of aquatic fauna, while creating opportunity for recolonization and range expansion at a later time. The post-glacial secondary contact hypothesis has been used to explain similar genetic patterns in other molluscan taxa, including slugs (Pinceel et al., 2005) and terrestrial gastropods (Davidson, 2000).

Sympatric speciation is another process that can generate genetically diverse sympatric lineages. It has been demonstrated that shifts in reproductive timing can result in sympatric speciation (allochrony) in invertebrate taxa, specifically those that have complex mating behaviors or periodicity, or those that utilize hosts (Wood & Guttman, 1982; Feder et al., 1993; Harrison & Bogdanowicz, 1995; Simon et al., 2000). *Cyprogenia aberti* possess a complex life cycle that provides many opportunities for allochronic speciation, where a shift in the timing of early reproductive events (i.e., gamete release, brooding, or larval release) could affect the success of their obligate parasitic larva. For example, larvae from animals that reproduce later in the reproductive season may be exposed to a different suite of potential fish hosts (Graf, 1997). If allochrony is a speciation mechanism in *C. aberti*, it does not appear to be a recent event, as evident from the non-sister clade pattern of the Ouachita groups. Instead, allochrony may have occurred within the Ouachita system, followed by range expansion of the two new lineages into other Highland drainages. Additional life history, geological, and perhaps nuclear gene sequences and molecular clock data will be needed to determine which speciation mechanism created the diversity in *C. aberti*.

Regardless of how the sympatric lineages in the Ouachita and White river systems were formed, the

question remains of how the color polymorphism could be maintained evolutionarily. Either the maintenance of color polymorphism within one species or the coexistence of sympatric species could result from frequency-dependent selection. In this model, host fish might avoid conglomerates after an initial encounter because of discomfort associated with glochidia attachment. Mussels producing a rare conglomerate color might, therefore, experience a selective advantage because fish would be less likely to have previous experience with that color. The selective advantage of a given color would increase with decreasing frequency in the population, protecting the polymorphism. It is necessary to establish several phenomena in order for this theory to gain credence, including the aversion of fishes to similar-colored conglomerates after an initial exposure. This theory also does not explain the very large genetic differences observed in the mitochondrial sequence data.

CONCLUSIONS

Gene trees are a critical first step when assessing biological diversity, but gene trees may be suspect if organisms have complex traits, such as unusual modes of genetic inheritance. We advocate that a comparative approach is the best method to assess biological diversity in these under-studied taxa. In general, we found congruence among the different data sets for *Cyprogenia aberti*. The congruence of conglomerate color and the mitochondrial gene tree appears to support separate nuclear lineages in *C. aberti* without direct evidence from a nuclear marker. In invertebrates, color morphs associated with mitochondrial lineages are not uncommon, and these data have been used to identify species even when color forms are otherwise morphologically and ecologically indistinguishable (Tarjuelo et al., 2004). If this precedent is followed, up to five species could be described in the *C. aberti* complex. Within the Ouachita River, two sympatric species exist: the red conglomerate producers (cf. Fig. 3, clade A) and the brown conglomerate producers (cf. Fig. 3, clade B). A similar division occurs in the White River, with red conglomerate producers and brown conglomerate producers co-occurring in the St. Francis River (Fig. 3). Future biological surveys of rivers in the White River system will be needed to determine these two species' ranges. Lastly, the upper Arkansas River contains a single species that produces white conglomerates.

In contrast to conglomerate color, the data on host fish indicate that there are no differences in host utilization among sympatric red and brown conglomerate forms within the Ouachita and White systems.

These data do not support, but also do not falsify, the hypothesis of sympatric species. Both genetic differences and host compatibility differences among geographic populations are consistent with limited gene flow and the possibility of several species within the *Cyprogenia aberti* group. These geographically separated *C. aberti* clades are not ecologically exchangeable. This conclusion is based on genetic divergence among lineages, difference in reproductive traits, such as conglutinate morphology, and preliminary data on host fish preference. Finally, the corroboration among different data sets suggests that *C. aberti* is a species complex that contains cryptic biological diversity. This diversity will be formally described in a forthcoming manuscript.

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APPENDIX 1. Voucher information for specimens sequenced in this study. GenBank accession numbers for the first subunit of cytochrome oxidase *c* (COI) and the first subunit of the NADH dehydrogenase (ND1) genes, respectively, are in parentheses. Voucher specimens are deposited at the University of Alabama Unionid Collection (UAUC) and are listed by museum accession numbers. Locality information and GenBank accession numbers for other species are found in Serb (2006).

Cyprogenia aberti:

Fall River (1). U.S.A. Kansas: Wilson Co., University of Alabama Unionid Collection [UAUC] 1646 (DQ494735, DQ494711), UAUC 1647 (DQ494728, DQ494708).

St. Francis River (3). U.S.A. Missouri: Wayne Co., UTM 15 722800E 4123900N (Patterson quad), B1 (DQ667060, DQ663617), B2 (DQ667061, DQ663618), B3 (DQ667062, DQ663619), B4 (DQ667063, DQ663620), B5 (DQ667064, DQ663621), R6 (DQ667065, DQ663622), R8 (DQ667066, DQ663623).

Buffalo River (4). U.S.A. Arkansas: Newton Co., UAUC 1650 (DQ494730, DQ494710).

Strawberry River (5). U.S.A. Arkansas: Sharp Co., UAUC 1652 (DQ494744, DQ494719), UAUC 2726 (DQ494745, DQ494720).

Current River (6). U.S.A. Arkansas: Clay Co., UAUC 1537 (DQ494727, DQ494707).

Black River (7). U.S.A. Arkansas: Randolph/Lawrence Co., UAUC 1648 (DQ494729, DQ494709), UAUC 2724 (DQ494742, DQ494717). Missouri: Butler Co., UTM 15 732729E 4071085N, M-mitotype (DQ663624).

White River (8). U.S.A. Arkansas: Woodruff/Jackson/White Co., UAUC 1654 (DQ494736, DQ494712).

Ouachita River (9). U.S.A. Arkansas: Montgomery Co., UTM 15 434359E 3828410N (Mount Ida quad), B1 (DQ667054, DQ663611), B2 (DQ667055, DQ663612), B3 (DQ667056, DQ663613), R5 (DQ667057, DQ663614), R6 (DQ667058, DQ663615), R7 (DQ667059, DQ663616).

Saline River (10). U.S.A. Arkansas: Saline Co., UAUC 76 (DQ494733, AY158749).

Caddo River (11). U.S.A. Arkansas: Pike Co., UAUC 1837 (DQ494739, DQ494715), UAUC 1838 (DQ494743, DQ494718). Arkansas: Clark Co., UAUC 2381 (DQ494741, DQ494716), UAUC 2383 (DQ494749, DQ494724), UAUC 2384 (DQ49447, DQ494722).

Cyprogenia stegaria:

Clinch River. U.S.A. Tennessee: Hancock Co., UAUC 1499 (AY654992, AY655089), UAUC 1500 (DQ494726, DQ494706).